Geldanamycin, an Inhibitor of Hsp90, Blocks Cytoplasmic Retention of Progesterone Receptors and Glucocorticoid Receptors via Their Respective Ligand Binding Domains

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Mudit Kakar,[1] Charu Kanwal,[1] J. Rian Davis,[1] Henan Li,[1] and Carol S. Lim[1]

[1]University of Utah, Department of Pharmaceutics and Pharmaceutical Chemistry, 421 Wakara Way #318, Salt Lake City, UT 84108

ABSTRACT

Steroid hormone receptors (SHRs), such as glucocorticoid receptors (GR) and progesterone receptors (PR), are shuttling proteins that undergo continuous nuclear import and export. Various mechanisms have been proposed to explain the localization of SHRs. It has been suggested that the ligand-binding domain (LBD) of SHRs is important in determining the subcellular localization. We have studied the localization of GR-LBD and PR-LBD alone, as well as of full-length GR and PR in the presence of geldanamycin (GA), a benzoquinoid ansamycin that specifically inhibits heat shock protection (Hsp90), using transient transfections and fluorescent microscopy. Our studies have indicated that GR-LBD and PR-LBD are retained in the cytoplasm via interaction with Hsp90. It was observed that in the unliganded state, treatment with GA translocates these LBDs to the nucleus. Similar results were obtained for full-length PR and GR. Additionally, it was found that after ligand induction, GA accelerated reexport of SHRs after ligand washout, implicating Hsp90 in nuclear retention of SHRs in the washout state. We also propose that a recently found "export" signal present in the LBD of SHRs is involved in interactions with Hsp90 and hence cytoplasmic retention of these receptors. After ligand induction, Hsp90 also may play a role in nuclear retention of SHRs following hormone washout.

KEYWORDS: Hsp90, ligand binding domain, progesterone receptor, glucocorticoid receptor, geldanamycin, cytoplasmic retention

INTRODUCTION

Steroid hormone receptors (SHRs) are shuttling proteins that undergo continuous nuclear import and export. Diverse SHRs localize differently in subcellular compartments in the unliganded state. For example, estrogen receptors (ER) are predominantly nuclear, progesterone receptors (PR) and mineralocorticoid receptors (MR) are nuclear with some extent of cytoplasmic localization, and glucocorticoid receptors (GR) and androgen receptors (AR) are mainly located in the cytoplasm.[1] These receptors localize in different compartments because of the disparity in their import/export kinetics.[2] If the import is rate limiting, the SHRs get exported faster, resulting in a net cytoplasmic localization. On the other hand, if the export is slower than the import, accumulation of the receptor in the nucleus results.

The reasons why SHRs exhibit predominantly nuclear or cytoplasmic localization in the unliganded state have been investigated by examining putative export and import receptors for these receptors. Crm1 (exportin 1), the classical export receptor that recognizes leucine-rich export signals,[3,4] is the best-characterized export receptor and has been implicated for some steroid receptors.[5-7] However, this protein's extent of contribution to the export of SHRs is still uncertain. The use of the Crm1 inhibitor leptomycin B (LMB) has been complicated by the fact that in addition to directly inhibiting Crm1,[8] LMB arrests the cell cycle.[9] Calreticulin (CRT), an endoplasmic reticulum resident protein, might also be involved in steroid receptor export,[10,11] but most likely only under acute cellular stress.[12] Phosphorylation of certain residues in the steroid receptors via protein kinases has also been implicated in steroid receptor export.[13-15] For example, phosphorylation of Ser 226 in GR and Ser 294 in PR has been reported to be involved in cytoplasmic transport.[16] However, the export receptor mediating these processes has not yet been defined. Recently, a signal that mediates nuclear export and is active in the absence of ligand was identified in AR. This signal is localized in the ligand-binding domain (LBD) of AR and is thought to mediate export of ER and MR as well.[17]

Nuclear localization of unliganded receptors is thought to be mediated by a classical nuclear localization signal (NLS), usually either bipartite or tripartite in nature, with basic (K or R) amino acids. These NLSs are usually found in the steroid receptor DNA binding domain or the hinge domain[18-20] and are imported by importin α.[20,21] The LBD is thought to contain a second NLS, at least for GR, that is activated upon addition of ligand.[20] Many groups have implicated the LBD

Corresponding Author: Carol S. Lim, University of Utah, Department of Pharmaceutics and Pharmaceutical Chemistry, 421 Wakara Way #318, Salt Lake City, UT 84108. Tel: (801) 587-9711; Fax: (801) 585-3614; E-mail: carol.lim@pharm.utah.edu

of steroid receptors in the subcellular distribution of these receptors. For example, removal of the entire LBD from GR resulted in receptors that were nuclear in the unliganded state.[21]

Saporita et al[17] recently hypothesized that there is an export signal in the LBD of AR that can mediate export independent of the Crm1 pathway. The nuclear export signal (NES) was hypothesized to consist of helixes 5 through 8 of the LBD and a highly conserved β turn between these helixes. The authors showed that a similar NES was also found in ER and MR. This NES did not exhibit a high amino acid homology with the AR NES but was similar in structure. The NES was shown to be necessary and sufficient to cause AR export. The authors concluded that this motif in the LBD is important in determining subcellular localization of SHRs. Since the LBD of steroid receptors is known to interact with Hsp90,[22,23] we investigated the role of Hsp90 in cytoplasmic localization (or export) of GR and PR (progesterone receptor [PRB] isoform) in the unliganded state. Additionally, after GR and PR were driven into the nucleus with ligand, the export of these receptors after hormone washout was also studied. The role of Hsp90 in nuclear retention of GR and PR after ligand washout was studied under these conditions.

Materials and Methods

Construction of Plasmids

GR

GR was tagged with enhanced green flourescent protein (EGFP) using the following procedure. Polymerase chain reaction (PCR) was used to selectively amplify the full-length GR of a green flourescent protein (GFP)-GR plasmid (rat C656G GR plasmid)[24] using 5′-AATTCTCGAGACTCCAAAGAATCCTTAGCTCCC-3′ as the forward primer and 5′-TTGGTACCTCATTTTTGATGAAACAGAAGCTTTTTG-3′ as the back primer. The PCR fragment was then digested with *Xho*I and *Kpn*I. pEGFP-C1 was also digested with these restriction endonucleases and ligated with the *Xho*I/*Kpn*I cut GR fragment.

PR-LBD

The LBD of PR was extracted by performing PCR on EGFP-PRB[4] using the forward primer 5′-GCGCGGTACCGTCAGATTGTGAGAGCACTGGA-3′ and the back primer 5′-GCGCGGATCCCAGTTATCTAGATCCGGTGGATCC-3′. The LBD product was digested with *Kpn*I and *Bam*HI. EGFP-C1 was then cut with *Kpn*I and *Bam*HI, calf intestinal phosphatase (CIP) treated, and ligated to the LBD. Next, EGFP-PR-LBD was cut with *Age*I and CIP treated. EGFP-C1 was digested with *Age*I and *Xma*I. The EGFP fragment was then ligated to the LBD constructs to make the double EGFP-tagged PR-LBD plasmid.

GR-LBD

The LBD of GR was extracted by performing PCR on GFP-GR plasmid[24] using forward primer 5′-GCGCGGTACCGCAGGAGTCCAAAGACACTTCG-3′ and back primer 5′-GCGCGGATCCGAAGATCGTCAGCATTACA-3′. This product was then inserted into EGFP-C1. The resulting plasmid, EGFP-GR-LBD, was then cut with *Age*I and CIP treated. EGFP-C1 was digested with *Age*I and *Xma*I, and the EGFP fragment was then ligated to the LBD constructs to make the double EGFP-tagged GR-LBD plasmid.

$GR^{621\text{-}695}$ (Numbering Based on Rat GR, Entrez Protein Locus NP_036708)

PCR was performed on GFP-GR using forward and backward primers 5′-AATTCTCGAGTTCTCATGGCATTTGCCTTGGGTTG-3′ and 5′-AAGGTACCCAGACCTTCCTTAGGAACTGAGG-3′, respectively. The product was digested with *Xho*I and *Kpn*I and inserted into the similarly cut EGFP-C1 vector.

$PR^{758\text{-}832}$ (Numbering Based on Human PR, Entrez Protein Locus AAS00096)

PCR was performed on EGFP-PRB using forward and backward primers 5′-GCGCAGATCTCTCATTCAGTATTCTTGGATGAGC-3′ and 5′-GCGCGAATTCCCCTTCCAAAGGAATTGTATTAAGAA-3′, respectively. The product was digested with *Eco*RI and *Bgl*II and inserted into the similarly cut EGFP-C1 vector.

$PR^{1\text{-}757}$

PCR was performed on hPRB (human) to obtain PR without the "export" motif using forward primer 5′-GCGCAGATCTGGCATGGACGAGCTGTACAAGTC-3′ and back primer 5′- GCGCGAATTCGCTCATCCAAGAATACTGAATGAGAG-3′. The product was digested with *Bgl*II and *Eco*RI and inserted into the similarly cut EGFP-C1 vector.

PR NLSc Mutant

The constitutively active NLS (NLSc) in EGFP-PRB was inactivated by replacing K638A, K640A, and K641A using the QuikChange Site-Directed Mutagenesis Kit by Stratagene (La Jolla, CA) with forward primer 5′-CTCTGACTTTATTGAACGCTGCAAATGCTCGACCTCCAAGGACCATGCCAGCC-3′ and back primer 5′-CTGGCATGGTCCTTGGAGGTCGAGCATTTGCAGCGTTCAATAAAGTCAGAG-3′.

PR NLSc Mutant$^{1\text{-}757}$

EGFP-PRB plasmid that had the NLS inactivated and was missing the export motif was constructed by conducting PCR on the EGFP-PRB NLSc mutant using the same primers as used for the PR$^{1\text{-}757}$ above.

GR$^{1\text{-}623}$

The plasmid encoding EGFP-GR without the export motif was constructed by using conducting PCR on EGFP-GR with primers 5′-AATTCTCGAGACTCCAAAGAATCC-TTAGCTCCC-3′, which contains the *Xho*I site, and 5′-GTACTCATGGATGTTTCTCATGGCAGGTACCTT-3′, which contains the *Kpn*I site. This PCR fragment contains GR but excludes the export motif. This fragment and pEGFP-C1 were independently digested and then ligated together.

Hsp90 Plasmid

cHsp90 (chicken) was a gift from Dr David Toft. It was cloned into a mammalian expression vector by extracting the fragment using PCR. The primers used were forward primer 5′-GCGCCGCTAGCGTTTAACTTTAAGAAGG-AGATATACATATG-3′ and back primer 5′-GCGCGAAT-TCGTGGTGGTGGTGCTCGAGTGCG-3′. The fragment obtained was digested with *Nhe*I and *Eco*RI and inserted into pEGFP-C1, which was digested with the same enzymes and CIP treated. EGFP-C1 was used as an expression vector only. The resulting plasmid does not contain EGFP.

Construction of PR plasmid (known as EGFP-PRB) and EGFP-EGFP has been previously described.[4]

All the insertions and mutations in the above constructs were verified by DNA sequencing (HSC Core Research Facilities, University of Utah).

Cell Culture

For these studies we used 1471.1 cells (murine adenocarcinoma cells that do not express endogenous PR) derived from a C127 cell line.[4] The cells were maintained in Dulbecco's modified eagle's medium (DMEM; GIBCO BRL, Grand Island, NY) with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), penicillin streptomycin (100 U/mL, GIBCO BRL), gentamicin (0.5 mg/mL, Hyclone), and L-glutamine (2 mM, Hyclone) in a 5% CO_2 incubator at 37°C.

Transient Transfections

Transient transfections of 1471.1 cells were performed by electroporation as previously described.[4] Briefly, 5×10^6 cells were transfected with 2 μg (or amounts indicated) of each of the desired plasmid(s) and 8 μg of carrier DNA, pGL3 basic (Promega, Madison, WI), to make 10 μg of total DNA. Transfections were performed using an Electrosquare Porator ECM 830 Electroporation system (BTX, San Diego, CA) at a voltage of 140 V, 10 msec, and 3 pulses in a total of 100 μ of cold plain DMEM. After a 5- to 10-minute recovery on ice, the electroporated cells were diluted with complete phenol red-free DMEM (10% FBS charcoal/dextran treated, Hyclone, L-glutamine, penicillin-streptomycin, and gentamicin) and plated on a clear coverglass (Corning no 1, 22 mm^2) in 6 well plates or in live cell chambers (Lab-tek chamber slide system, 2 mL, Nalge NUNC International, Naperville, IL). These cells were then incubated in a 5% CO_2 incubator at 37°C for 18 to 24 hours.

Microscopy

Approximately 24 hours after electroporation, protein expression of plasmids was examined by fluorescence microscopy in fixed or living cells.[4,25] An Olympus IX701F inverted fluorescence microscope (Scientific Instrument Co, Aurora, CO) with a high-quantity narrow band GFP filter (excitation filter set HQ480/20nm; emission filter set HQ510/20 nm; with beamsplitter Q4951p) from Chroma Technology Corp (Brattleboro, VT) was used. Cells were photographed at a magnification of 40× or 60× using an F-View Monochrome CCD camera (Olympus, Hamburg, Germany). To minimize photobleaching of the EGFP chromophore, cells were imaged using neutral-density filters that transmit 25% of the total light, and short exposure times of 500 ms were used.

For live cell imaging, a Nevtek ASI 400 Air Stream Incubator (Burnsville, VA) with variable temperature control was used to maintain the microscope stage and the live cell chambers at 37°C.

Chemicals

Transfected 1471.1 cells were grown in phenol red-free DMEM with 10% charcoal-stripped FBS for 24 hours prior to addition of geldanamycin (GA) or vehicle. The medium was refreshed prior to the addition of GA (InvivoGen, San Diego, CA), an inhibitor of Hsp–steroid receptor interaction dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA) or vehicle where specified. Control cells were treated with the same volume of vehicle, DMSO, for the same time periods. The ligands dexamethasone (Sigma, St Louis, MO) and the synthetic progestin R5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; DuPont NEN, Boston, MA) were used to induce GR and PR, respectively.

Data Analysis

All images were analyzed by analySIS software (Soft Imaging System GmbH, Lakewood, CO), as described previously. Average intensities were calculated to overcome the variations due to differences in the shape and spread of the cells. To calculate the relative intensity of the nucleus to the cytoplasm, the average nuclear intensity (nuclear intensity divided by nuclear area) was calculated for each cell and divided by the average cytoplasmic intensity (cytoplasmic intensity divided by area of cytoplasm).[26]

Statistical Analysis

Statistical analysis of the data was performed with GraphPad InStat version 3.02 software (GraphPad Software, Inc, San Diego, CA). The difference in fluorescence intensity between samples was tested using the Wilcoxon rank sum (Mann-Whitney) test for 2 independent samples.

RESULTS

The export capability of complete LBDs of these SHRs was tested by creating double EGFP fusion proteins with a PR or GR LBD. These doubly tagged constructs (~86 kDa) were designed to make sure they were over the size range that can still undergo passive diffusion (40-60 kDa) into the nucleus via the nuclear pore complex.[3] As shown in Figure 1A, PR-LBD showed both nuclear and cytoplasmic distribution, while GR-LBD (Figure 1B) showed predominantly cytoplasmic localization. The control containing EGFP alone (Figure 1C, double EGFP) localized in the nucleus and cytoplasm.

Hsp90 is known to interact with LBDs of both PR and GR.[22,23,27,28] Thus, the effect of the Hsp90 inhibitor GA was tested. GA is a benzoquinoid ansamycin that specifically occupies the nucleotide binding site on Hsp90 and prevents the switch to its ATP-bound conformation, which is required for binding to steroid receptors.[29,30] GA can also directly block binding of p23 to Hsp, leading to arrest of PR assembly.[31] As seen in Figure 2, after a 12- or 24-hour treatment with 1 µg/mL GA, GR-LBD appeared more nuclear (Figures 2B, 2D) than it did with vehicle treatments (Figures 2A, 2C), although complete nuclear localization was not seen. For GR-LBD, the average nuclear-to-cytoplasmic ratios were higher for 12-hour GA treatment than for 12-hour vehicle treatment, and this difference was statistically significant ($P < .0001$). GA had no effect on PR-LBD, perhaps since PR-LBD is somewhat nuclear to begin with (data not shown).

The effect of GA was then tested on full-length GR and PR. In the absence of GA, full-length PR localized mostly in the nucleus but had some cytoplasmic distribution (Figures 3E, 3G), while full-length GR was predominantly cytoplasmic (Figures 3A, 3C), as expected.[24,32] GR appeared more nuclear with either 12-hour (Figure 3B) or 24-hour (Figure 3D) 1 µg/mL GA treatment compared with the control treated with vehicle (DMSO) for the same time (Figures 3A and 3C for 12 and 24 hours, respectively). This difference was statistically significant at the 12-hour time point ($P = .0026$). PR also appeared slightly more nuclear in the presence of 1 µg/mL GA (Figures 3F and 3H for 12 and 24 hours, respectively) than in the presence of the control (Figures 3E and 3G for 12 and 24 hours, respectively), although this difference was not statistically significant ($P > .05$) for the number of cells analyzed. However, since PR has a constitutively active NLS,[33] this signal drives most PR into the nucleus[32] regardless of GA. To overcome this problem, the NLS in PR was inactivated (PR NLSc mutant) and tested with GA. Indeed, this PR NLSc mutant was cytoplasmic in the unliganded state at 12 hours (Figure 3I) and 24 hours (Figure 3K) for cells treated with the vehicle but became more nuclear for cells treated with GA (Figures 3J and 3K for 12 and 24 hours, respectively). This difference was also statistically significant at the 12-hour time point ($P < .05$). In addition to using low-dose GA

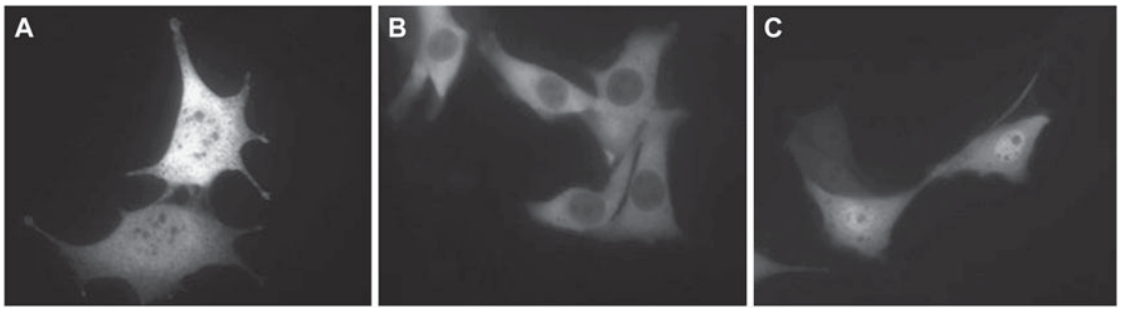

Figure 1. Ligand binding domain of PR/GR is involved in cytoplasmic subcellular localization. PR-LBD and GR-LBD were tagged to EGFP-EGFP. Localization of PR-LBD (1A), GR-LBD (1B), and EGFP-EGFP (1C) is shown. All are tagged with EGFP-EGFP; single-tagged EGFP constructs showed similar results (data not shown). PR indicates progesterone receptor; GR, glucocorticoid receptor; LBD, ligand binding domain; EGFP, enhanced green flourescent protein.

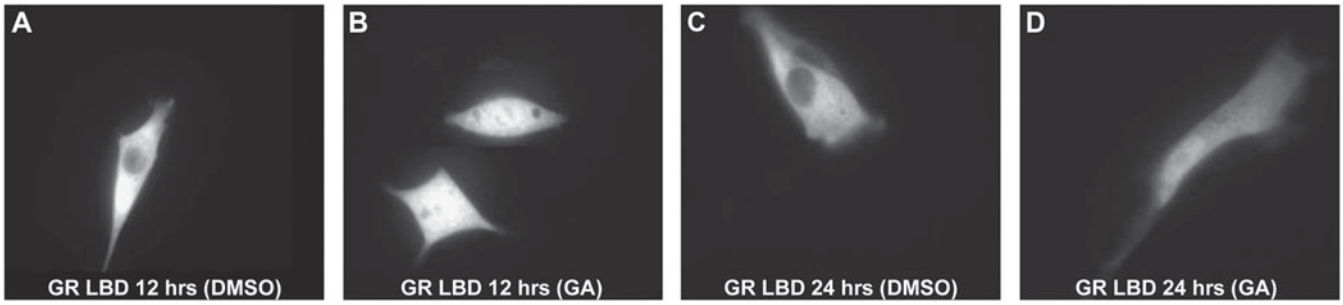

Figure 2. Effect of GA on localization of unliganded GR-LBD (double EGFP tagged). Cells were treated with 1 μg/mL of GA for a period of 12 hours (2B) or 24 hours (2D), as specified. GA seemed to cause increased nuclear accumulation of the construct as compared with DMSO vehicle (2A, 2C). GA indicates geldanamycin; GR, glucocorticoid receptors; LBD, ligand binding domain; EGFP, enhanced green flourescent protein; DMSO, dimethyl sulfoxide.

for long durations, as done here and also in others' studies,[34-36] we tested high-dose GA for a short duration[31,37,38] on the GR construct. As shown in Figure 4A, high-dose/short-duration GA treatment (25 μM, 20 minutes) on full-length GR also results in more nuclear localization of GR; these results were also statistically significant ($P < .05$) compared with the control, where the vehicle (DMSO) was added instead of GA (Figure 4B).

In an attempt to "force" more PR into the cytoplasm, Hsp90 was overexpressed with PR. Hsp90 might have had a slight effect on PR (making it more cytoplasmic, Figure 5B), but this difference was not statistically significant for the number of cells tested (Figure 6, fourth set of bars). These results were not surprising because PR has a strong constitutive NLS, and any effects of Hsp90 are overridden.

A summary of data represented in Figures 2, 3, 4, and 5 appears in Figure 6, which shows the effect of GA on GR-LBD, GR, PR, PR + Hsp90, and PR NLSc mutant. Statistically significant differences were found for GR-LBD, GR, and PR NLSc mutant.

The putative NES motif in the LBD of AR defined by Saporita et al[17] was present between amino acids 743 and 817—a stretch of amino acids conserved in many steroid receptors. A deletion mutant of AR lacking the export motif showed predominantly nuclear localization, underscoring its importance in determining nucleocytoplasmic distribution. A similar sequence was found in PR, between amino acids 758 and 832, and in GR, between amino acids 621 and 695 (Figure 7A). The LBDs of steroid receptors would be expected to be conserved since they all recognize ligands based on steroid structure. Localization of constructs containing export motifs (defined by Saporita et al[17]) alone ($PR^{758-832}$ and $GR^{621-695}$) was found to occur in the cytoplasm (Figure 7B). These motifs showed some spotted fluorescence, suggesting the degradation of these nonnative protein fragments via molecular chaperones.[39]

PR and GR were truncated to exclude the amino acid sequence starting from the export motif and moving beyond (PR^{1-757} and GR^{1-623}), and the localization of these constructs was examined using fluorescence microscopy (Figures 8B, 8F). Both the constructs were observed to be distributed mainly in the nucleus, compared with full-length PR (nuclear and cytoplasmic) and GR (mostly nuclear), as shown in Figures 8A and 8E, respectively. To test whether the change in localization after making LBD truncations was due to better exposure of the constitutive NLS in PR, PR NLSc mutant was truncated to exclude the export motif. The subcellular localization of this construct (PR NLSc mutant$^{1-757}$) was determined (Figure 8D). This NLSc mutant, lacking the export motif, showed a more nuclear distribution than the untruncated NLSc mutant did (Figure 8C).

Lastly, we studied the role of Hsp90 in nuclear *retention* of SHRs after ligand withdrawal. Tago et al showed that in COS7 cells, after induction with hormone, GR translocated to the nucleus; after hormone washout, GR was exported back to the cytoplasm. Interestingly, GA was found to accelerate the export of GR back to the cytoplasm.[40] This suggests that Hsp90 plays a role in nuclear retention of GR. To confirm this, we performed similar studies with GA for PR and GR in our system. PR was treated with 1 nM R5020 and driven into the nucleus (Figure 9A). Following ligand washout, cells were incubated with GA (1 μg/mL). Control cells were treated with the vehicle DMSO for the same time periods. Export was studied over a period of 24 hours at the time points of 12 and 24 hours. In the presence of GA, retention of PR in the nucleus was reduced (and cytoplasmic translocation was increased) compared with control cells treated with DMSO (the experiment was conducted 5 times). There was a statistically significant difference in subcellular localization in the constructs in the presence and absence of GA at 12 hours ($P = .0010$) but not at 24 hours ($P > .05$). The lack of significance at 24 hours was not unexpected, considering that PR has a constitutively active NLS that causes PR to be

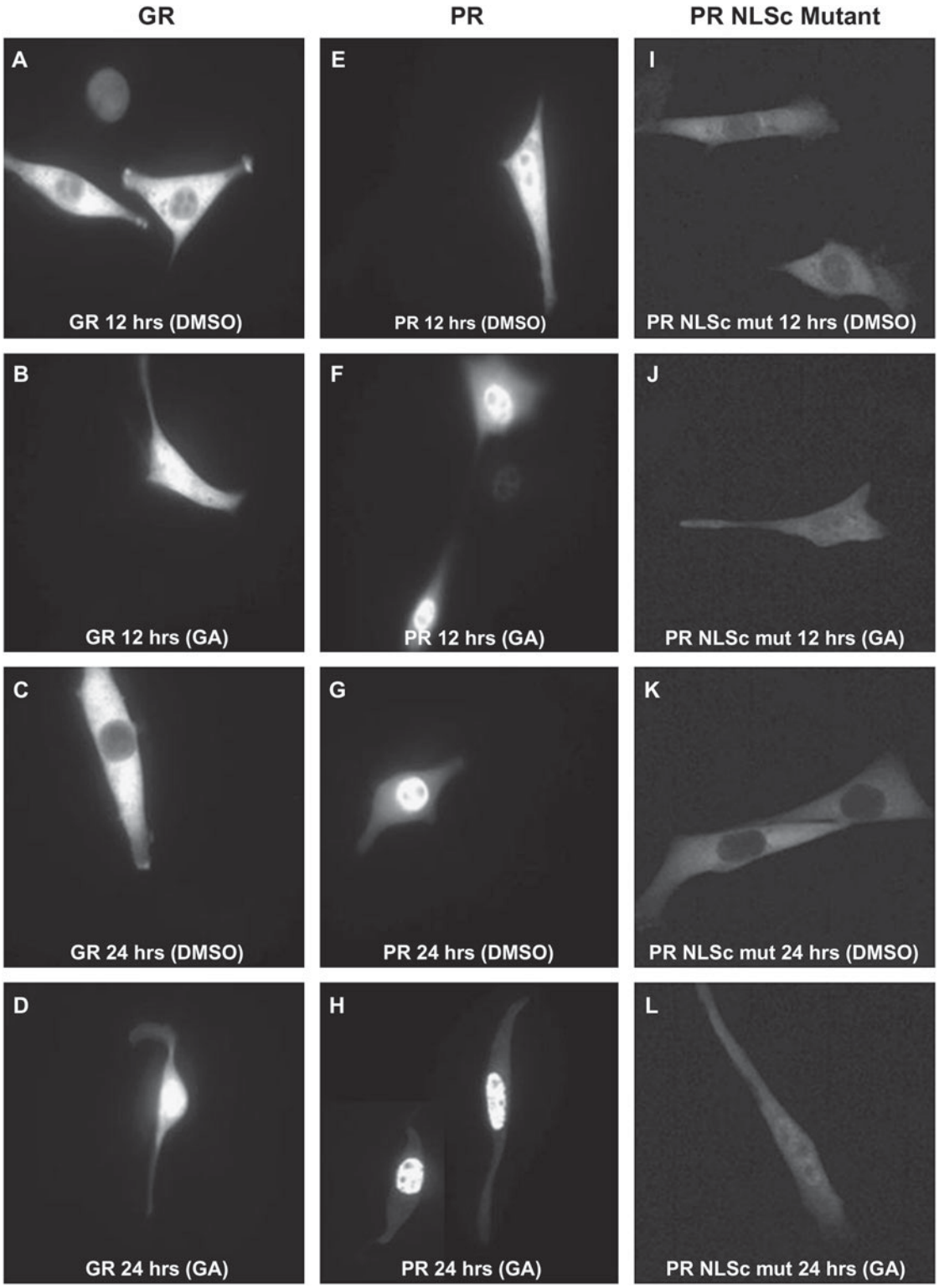

Figure 3. Effect of GA on localization of unliganded PR/GR. Cells were treated with 1 µg of GA for a period of 12 or 24 hours, as specified. GA caused increased nuclear accumulation of GR at 12 hours (3A vs 3B) and 24 hours (3C vs 3D). For PR, a similar effect was seen, although it was very subtle (3E, 3F, 3G, 3H). PR NLSc mutant was more nuclear in the presence of GA at both 12 and 24 hours (3I, 3J, 3K, 3L). GA indicates geldanamycin; PR NLSc mut, progesterone receptor with the constitutively active nuclear localization signal mutated; GR, glucocorticoid receptor; DMSO, dimethyl sulfoxide; mut, mutant.

mostly nuclear even in the absence of ligand.[32] For studies with GR, GA was found to reduce the retention of GR in the nucleus and to increase cytoplasmic translocation as compared with control cells treated with DMSO. GR localization was significantly different on treatment with GA at 12 hours ($P < .0001$) and at 24 hours ($P = .0145$).

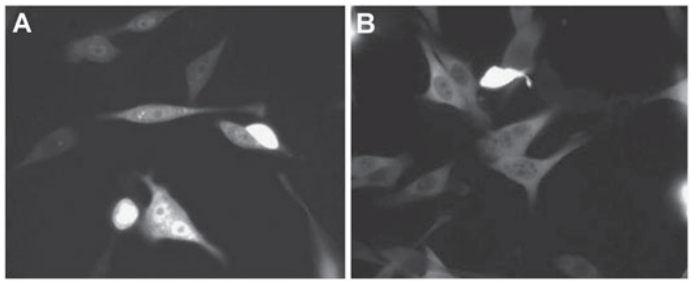

Figure 4. Effect of high-dose/low-concentration GA on localization of full-length GR. Cells were treated with 25 μ GA for 20 minutes. GA caused increased nuclear accumulation of the receptor. The difference in nuclear localization of full-length GR between GA (4A) and DMSO (4B) (vehicle) was statistically significant. GA indicates geldanamycin; GR, glucocorticoid receptor; DMSO, dimethyl sulfoxide.

DISCUSSION

Previous studies have shown that the LBD is important for determining subcellular localization. Wan et al found that exchanging the LBD of PR with that of GR led to a more cytoplasmic localization of chimeric PR. Similarly, exchanging the LBD of GR with that of PR caused the chimeric GR to be more nuclear as compared with the wild-type receptor.[41] Kino et al suggested that an altered LBD may impede the exit of GR from the nucleus.[21] These reports indicate that LBD plays an important role in determining the subcellular localization of the receptor and that helices 1 to 5 could likely determine localization specificity.[21,41] Recently, Saporita et al[17] reported the presence of an export motif in the LBD of AR, ER, and MR that was necessary and sufficient for export. We have identified a comparable motif in PR and GR, present between amino acids 758 and 832 for PR and 621 and 695 for GR. This motif also lies within the LBD. The LBD of these receptors binds to heat shock proteins in the cytoplasm, which keeps them in a conformation suitable for steroid binding. A single region in the steroid receptor LBD that binds to Hsps has not been defined,[23] and

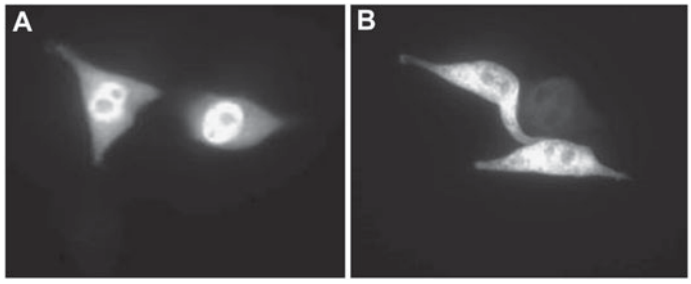

Figure 5. Effect of Hsp90 overexpression on export of PR. PR control localization (5A) as compared with localization with overexpression of Hsp90 (5B). Experiments were performed in triplicate. Hsp90 indicates heat shock protein 90; PR, progesterone receptor.

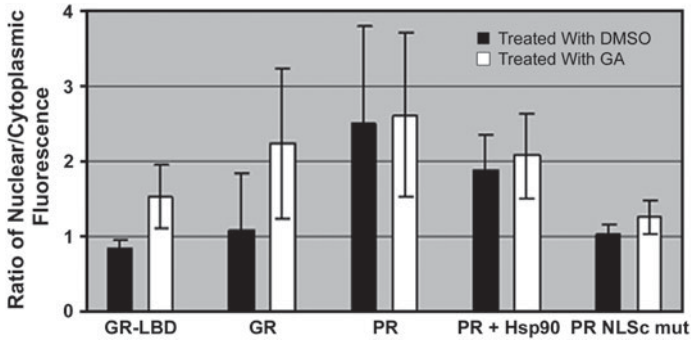

Figure 6. Effect of GA treatment on unliganded receptors. Cells transfected with GR-LBD (data from Figure 2); GR, PR, and PR NLSc mut (data from Figure 3); and PR cotransfected with Hsp90 (data from Figure 5) were treated with GA for 12 hours and the localization was examined. GA reduced the cytoplasmic localization of these proteins. Cytoplasmic localization of GR-LBD and GR was reduced considerably, while that of PR and PR overexpressed with Hsp90 was reduced only slightly. Data are presented as mean ± SD, n = 15 (experiments were conducted in triplicate). There was a considerable difference between the constructs treated with GA and those treated with DMSO in the case of GR-LBD ($P < .0001$) and GR ($P = 0.0026$). Although localization of unliganded PR induced with GA was slightly more nuclear than without GA, this difference was not significant for this number of cells ($P > .05$). Also, the PR constructs overexpressed with Hsp90 did not show any major difference on treatment with GA ($P > .05$). GA indicates geldanamycin; DMSO, dimethyl sulfoxide; GR, glucocorticoid receptor; LBD, ligand binding domain; PR NLSc mut indicates progesterone receptor with the constitutively active nuclear localization signal mutated.

it is possible that Hsps recognize a tertiary structure and not specific amino acids for interaction.[22] However, the amino acid region 556 to 659 in GR has been shown to be important for Hsp90 binding.[27,42] Since the NES defined by Saporita et al[17] falls within the LBD, we wanted to determine whether the NES, instead of acting as an export signal per se, was mediating cytoplasmic localization of the receptor via Hsp90. Removal of this export motif could lead to a more nuclear distribution, if Hsp90 cannot bind to it. Since Hsp90 functions in part as a cytoplasmic retention factor for steroid receptors, we investigated the role of Hsp90 in subcellular localization of PR and GR.

Wild-type truncated PR/GR, excluding the export motif, showed mainly nuclear localization. It is possible that the cytoplasmic retention of wild-type PR/GR is due to binding to heat shock proteins, as the minimal high-affinity binding region in GR that binds to Hsp90 is between amino acids 556 and 659,[27,42] which contains part of the export motif (Saporita et al[17]). Similarly, the region identified in PR for Hsp binding[23] encompassed the conserved region of the export motif. Heat shock proteins are known to form a heteromeric complex with SHRs in the cytoplasm. Hsps bind

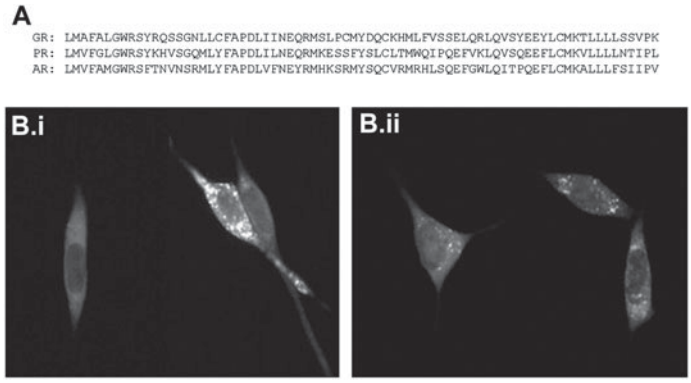

Figure 7. Localization of export motif. (A) Export motifs in PR and GR aligned with the NES motif described by Saporita et al in ARs.[17] (B) EGFP-EGFP-GR$^{621\text{-}695}$ (7B.i) and EGFP-EGFP-PR$^{758\text{-}832}$ (7B.ii) localize at least to some degree in the cytoplasm. GR (621-695) numbering corresponds to rat GR, PR (758-832) numbering corresponds to human PR, aR (743-817) numbering corresponds to human AR from Saporita et al. PR indicates progesterone receptors; GR, glucocorticoid receptor; AR, androgen receptor; NES, nuclear export signal; EGFP, enhanced green flourescent protein.

to the LBD of steroid receptors and maintain these receptors in a conformation susceptible to ligand binding.[43] It has also been suggested that these proteins are translocated as a complex with the steroid receptors on ligand induction.[38] However, the mode of export of heat shock proteins back into the cytoplasm is unknown.

GA causes destabilization of Hsp–steroid receptor complexes. GA's effect on the nucleocytoplasmic distribution of PR and GR in the absence of ligand was studied. The unliganded receptors localized mainly in the cytoplasm. However, adding GA to both PR and GR led to more nuclear localization, suggesting reduced cytoplasmic retention on inhibition of Hsp binding. The cytoplasmic localization of GR-LBD was also reduced on treatment with GA, indicating that LBD is involved in cytoplasmic retention via Hsp90. PR has a strong constitutive NLS that causes movement of the receptor to the nucleus regardless of GA. However, localization of PR NLSc mutant into the nucleus on induction with GA further suggests that LBD is involved in cytoplasmic retention via Hsp90. There is a possibility that GA may indirectly affect cellular transport as well because of the large number of Hsp90 clients. However, GA has not yet been shown to interact with import/export machinery and has been very commonly used in SHR transport studies.[35-39]

Nuclear localization of PR$^{1\text{-}757}$ and GR$^{1\text{-}623}$ (PR and GR with truncated export motif) implies that this motif is retained in the cytoplasm by some mechanism. PR NLSc mutant$^{1\text{-}757}$, lacking the export motif and the strong constitutive NLS, localized in the nucleus further, suggesting the

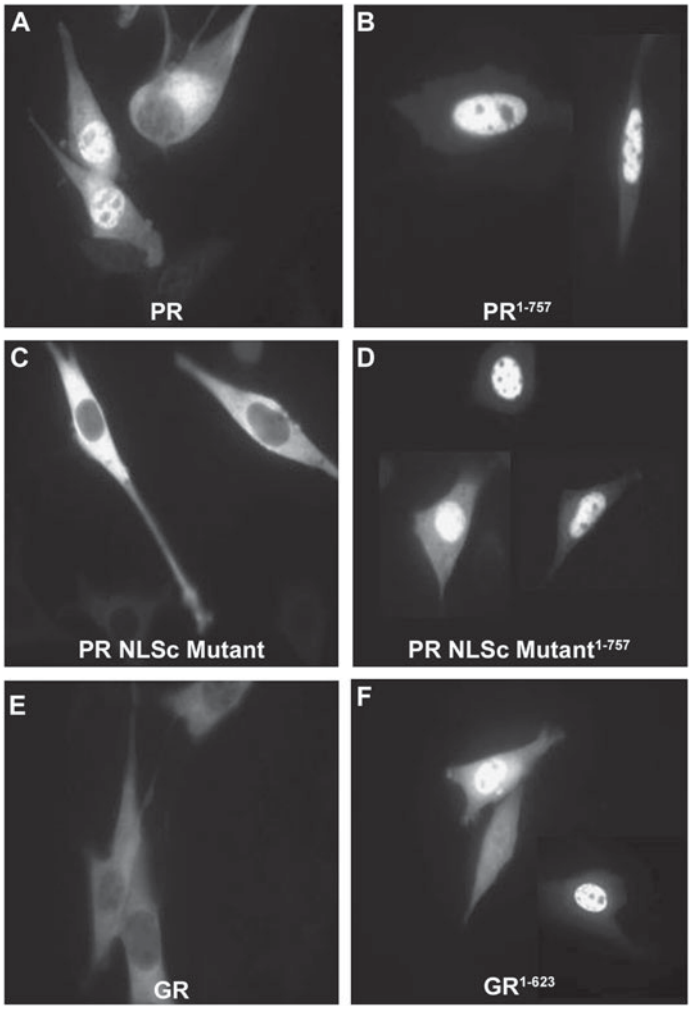

Figure 8. Effect of truncations on PR and GR localization. PR, PR NLSc mutant, and GR were truncated to exclude the export motif identified by Saporita et al[17] and beyond. In all cases, exclusion of the motif results in more nuclear localization. PR indicates progesterone receptor; PR NLSc mut, progesterone receptor with the constitutively active nuclear localization signal mutated; GR, glucocorticoid receptor.

involvement of the export motif in cytoplasmic retention. Since steroid receptors mainly bind to heat shock proteins in the cytoplasm, this export motif may play a central role in Hsp binding, and its removal leads to enhanced nuclear localization. Thus, the export motif, present in LBD, defined by Saporita et al is most likely involved in cytoplasmic retention of receptors via Hsp90, rather than being a true export signal sequence.[3] However, no single clearly demarcated region has yet been identified for binding to Hsp90.[23,44] Our studies show that in the absence of ligand Hsp90 is involved in cytoplasmic retention of PR and GR.

Interestingly, in the presence of ligand, Hsp90 is thought to be involved in the nuclear translocation and/or nuclear retention of GR. Several studies have shown that blocking of Hsp90 with GA leads to loss of nuclear translocation in the

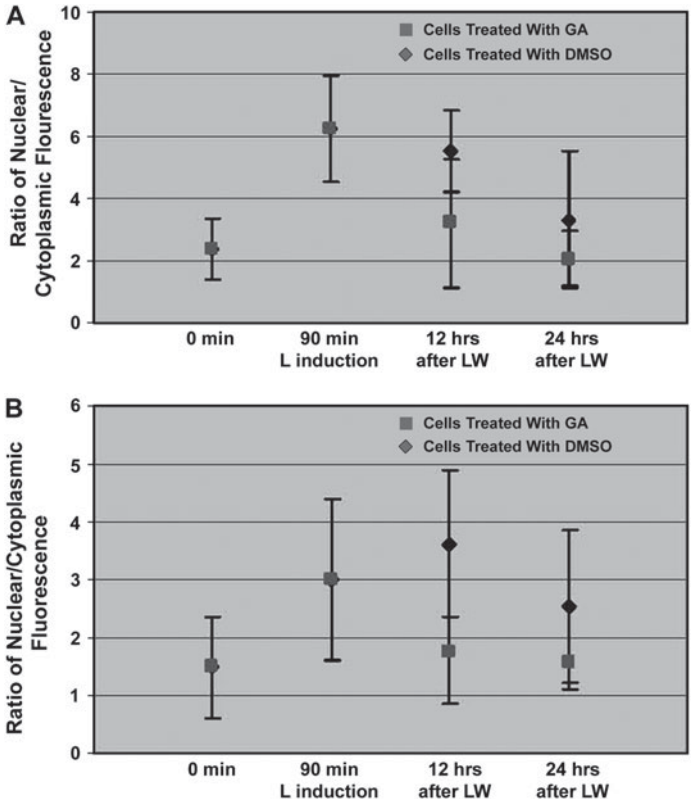

Figure 9. PR and GR export (after ligand induction) in the presence of GA. Export studies of PR and GR were performed 24 hours after transfection. Cells were induced with ligand for 90 minutes (1 nM R5020 for PR and 0.1 nM dexamethasone for GR) for 90 minutes. Ligand was washed off and cells incubated in fresh media in the presence of GA (1 μ/mL). Control cells were treated with the vehicle DMSO for the same time periods. Export was studied over a period of 24 hours at time points of 12 and 24 hours. (A) GA reduced the retention of PR in the nucleus and increased cytoplasmic translocation as compared with control cells treated with DMSO (experiment conducted 5 times). Data are presented as mean ± SD, n = 20. Mann-Whitney test supported a considerable difference in subcellular localization in the constructs in the presence and absence of GA at 12 hours ($P = .0010$) but not at 24 hours ($P > .05$). (B) GA reduced the retention of GR in the nucleus and increased cytoplasmic translocation as compared with control cells treated with DMSO. Data are presented as mean ± SD, n = 20. GR localization was significantly different on treatment with GA at 12 hours ($P < .0001$) and at 24 hours ($P = .0145$). L indicates ligand; LW, ligand withdrawal; GA, geldanamycin; DMSO, dimethyl sulfoxide; PR, progesterone receptors; GR, glucocorticoid receptors.

presence of ligand.[23,37,45] Tago et al found that after hormone induction followed by washout, GA accelerated the relocation of GR to the cytoplasm. This suggests a role for Hsp90 in nuclear retention of GR after ligand washout. Our studies (Figure 9) corroborate these findings as well. The cytoplasmic relocalization of PR and GR after ligand withdrawal was enhanced with treatment with GA (Figure 9), indicating that Hsp90 is somehow also involved in the nuclear retention of these receptors. If Hsp90 is involved both in export of SHRs and in nuclear retention after ligand withdrawal, additional associated proteins or a signaling mechanism may be necessary to stimulate these events. The protein p23, the smallest member of the Hsp machinery, has been shown to be involved in receptor-Hsp signaling interaction[31,33,45] and could be involved in this process.

Conclusion

Steroid receptors are shuttling proteins whose import in the unliganded state is well defined and well studied.[20,33,46-50] However, the export of steroid receptors has not been fully nailed down and has been proposed to occur by several different mechanisms. These include export via CRM1, via CRT, and via phosphorylation. We believe that the new export motif defined by Saporita et al interacts with Hsp90 and is retained in the cytoplasm via Hsp90. Indeed, studies with the Hsp90 inhibitor GA provide strong evidence of this. Hsp90 also seems to play a role in nuclear retention of SHRs after ligand washout. Export (and import) of proteins besides steroid receptors may occur via a piggyback mechanism with other molecular chaperones,[51-53] which adds to the complexity of studying SHR export.

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